Simultaneous determination of bosentan and glimepiride in human plasma by ultra performance liquid chromatography tandem mass spectrometry and its application to a pharmacokinetic study

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ABSTRACT

A sensitive and rapid ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method was developed to determine bosentan (BOS) and glimepiride (GPD) in human plasma simultaneously. Chromatographic separation was carried out on an Acquity UPLC BEH C18 column and mass spectrometric analysis was performed using a QTrap5500 mass spectrometer coupled with an electro-spray ionization (ESI) source in the positive ion mode. The MRM transitions of m/z 552.0 → 202.1 and m/z 491.2 → 125.9 were used to quantify BOS and GPD, respectively. This assay method has been fully validated in terms of selectivity, linearity, recovery and matrix effect, accuracy, precision and stability. The linearity of this method was found to be within the concentration range of 5–1000 ng/mL for BOS, and 2.5–500 ng/mL for GPD in human plasma. Only 1.5 min was needed for an analytical run. This assay was used to support a clinical study where multiple oral doses were administered to healthy Chinese subjects to investigate the pharmacokinetics of BOS and GPD.

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1. Introduction

Bosentan (BOS) is a potent nonpeptide dual endothelin receptor antagonist (ERA) with affinity for both endothelin A and endothelin B receptors. It decreases both pulmonary and systemic vascular resistance, thereby increasing cardiac output without increasing the heart rate [1,2]. The single-dose pharmacokinetics of BOS have been described previously and were recently reviewed [3,4]. In brief, BOS shows dose-proportional pharmacokinetics up to single oral doses of 600 mg and an oral bioavailability of 50%. After intravenous administration of a 250 mg dose, a volume of distribution of 18 L and a clearance of 8.2 L/h were determined [5]. The apparent half-life after oral administration of 125 mg was 5.4 h [6]. BOS is extensively metabolized in the liver by CYP3A4 and CYP2C9 enzymes to give three metabolites namely, the hydroxybosentan (HYBOS), phenol metabolite and hydroxyl-phenol metabolite [7,8].

Several methods are reported for the determination of BOS in different biological matrices [9,10]. Glimepiride (GPD) is a third generation sulfonylurea type oral hypoglycemic agent, which is widely used in the treatment of type 2 diabetes [11,12]. In clinical trials, GPD is now considered to be a safe and effective hypoglycemic agent that does not lead to a gain in body weight [13]. GPD is almost completely bioavailable from the gastrointestinal tract [14] and metabolized by CYP2C9 to hydroxy-glimepiride (M1), which is the rate-limiting step in its elimination process [15]. Several methods have been developed for the determination of GPD in human plasma by means of high performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) [16–20].

When BOS and GPD are used in combination in clinic, they may have many potential drug interactions as they are all CYP2C9 substrates. Even though various methods were reported in the literature for estimation of BOS and GPD individually or in combination with other drugs [21,22], no method had been reported for simultaneous estimation of these two drugs using ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS). Thus, it was essential to establish a high sensitive and more efficient assay for the simultaneous determination of BOS and GPD. In the present study, we developed an UPLC–MS/MS method for the simultaneous...
determination of BOS and GPD using losartan as an internal standard. This new method has been fully validated in terms of specificity, linearity, matrix effect and recovery, accuracy, precision and stability.

2. Experimental

2.1. Chemicals and reagents

BOS (purity 98.0%), GPD (purity 98.0%), and losartan (internal standard, IS, purity 98.0%) were obtained from Sigma (St. Louis, MO, USA). Formic acid was analytical grade and purchased from the Beijing Chemical Reagents Company (Beijing, China). Acetonitrile was of HPLC grade and was purchased from Merck Company (Darmstadt, Germany). HPLC grade water was obtained using a Milli Q system (Millipore, Bedford, USA). Blank plasma used in this study was supplied by the Second Affiliated Hospital of Wenzhou Medical University (Wenzhou, China).

2.2. UPLC–MS/MS conditions

Liquid chromatography was performed on an Acquity ultra performance liquid chromatography (UPLC) unit (Waters Corp., Milford, MA) with an Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm particle size) and inline 0.2 μm stainless steel frit filter (Waters Corp., Milford, USA). A gradient program was employed with the mobile phase, combining solvent A (0.1% formic acid in water) and solvent B (acetonitrile) as follows: 35% B (0–0.5 min), 35–80% B (0.5–1.0 min), 80–35% B (1.0–1.5 min). A subsequent re-equilibration time (1 min) should be performed before next injection. The flow rate was 0.45 mL/min and the injection volume was 10 μL. The column and sample temperature were maintained at 40 °C and 4 °C, respectively.

An AB Sciex QTRAP 5500 triple quadrupole mass spectrometer equipped with an electro-spray ionization (ESI) source (Toronto, Canada) was used for mass spectrometric detection. The quantitative analysis of BOS and GPD in human plasma was performed using multiple reaction monitoring (MRM) method. The dwell time was set to 300 ms for each MRM transition. The MRM transitions were m/z 552.0 → 202.1, m/z 491.2 → 125.9, and m/z 423.2 → 207.1 for BOS, GPD and IS, respectively (Fig. 1). The optimal MS parameters were as follows: capillary voltage 3.5 kV, desolvation line (DL) temperature 250 °C, heat block temperature 400 °C, nebulizing gas flow and drying gas flow were 3.0 L/min and 15.0 L/min, respectively. Data acquiring and processing were performed using analyst software (version 1.5, AB Sciex).

2.3. Standard solutions, calibration standards and quality control (QC) sample

The stock solutions of BOS and GPD used to make the calibration standards and quality control (QC) samples were prepared by dissolving 10 mg each compound in 10 mL methanol to obtain a concentration of 1.00 mg/mL of each compound. The stock solutions were further diluted with methanol to obtain working solutions at several concentration levels. Calibration standards and QC samples in plasma were prepared by diluting the corresponding working solutions with blank human plasma. Final concentrations of the calibration standards were 5, 10, 25, 50, 100, 250, 500 and 1000 ng/mL for BOS, and 2.5, 5, 10, 25, 50, 100, 250 and 500 ng/mL for GPD in human plasma, respectively. The concentrations of QC samples in plasma were 10, 200, and 800 ng/mL for BOS, and 5, 100, and 400 ng/mL for GPD, respectively. IS stock solution was made at an initial concentration of 1 mg/mL. The IS working solution (50 ng/mL) was made from the stock solution using acetonitrile for dilution. All stock solutions, working solutions, calibration standards and QCs were immediately stored at −80 °C.

2.4. Sample preparation

Before analysis, the plasma sample was thawed to room temperature. In a 1.5 mL centrifuge tube, an aliquot of 200 μL of the IS working solution (50 ng/mL in acetonitrile) was added to 100 μL of collected plasma sample. The tubes were vortex mixed for 1.0 min and spun in a centrifuge at 12,000 rpm for 10 min. The supernatant (10 μL) was injected into the UPLC–MS/MS system for analysis.

2.5. Method validation

Before using this method to determine BOS and GPD in clinical samples, the method was fully validated for specificity, linearity, sensitivity, precision, accuracy, recovery, matrix effect and stability according to the United States Food and Drug Administration (FDA) guidelines for the validation of a bioanalytical method [23].

A selectivity study is designed to investigate whether endogenous constituents and other substances existing in samples will interfere with the detection of analytes and IS. Selectivity was studied by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Calibration curves were prepared according to Section 2.3. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of analytes to IS versus the nominal concentration (x) of analytes with weighted (1/x²) least square linear regression. The lower limits of quantification (LLOQ) and limits of detection (LOD) were calculated based on signal-to-noise ratio of 10:1 and 3:1, respectively. LOD and LLOQ were defined as the analytes responses which yielded a signal to noise ratio of greater than 3 and 10, respectively, indicating that this method is sensitive for the quantitative evaluation of the analytes.

The extraction recovery was evaluated by comparing peak areas obtained from extracted spiked samples with those of the post-extracted spiked samples at corresponding concentrations. The extraction efficiency of the analytes was determined by analyzing six replicates of QC samples at three concentration levels.

The matrix effect was evaluated by comparing the peak areas of the post-extracted spiked QC samples with those of corresponding standard solutions. The matrix effect of the analytes was determined by analyzing six plasma samples at three concentration levels. The extraction recovery and matrix effect of IS were
determined using the same procedure at a single concentration of 50 ng/mL.

For the evaluation of intra-day precision and accuracy, six replicates QC samples were analyzed at three concentration levels on the same day. For the evaluation of inter-day precision and accuracy, three replicates of QC samples were analyzed at three concentration levels on six consecutive days. Precision was expressed as % relative standard deviation (RSD) and accuracy was expressed as % relative error (RE) between the measured and nominal value. The precision for QC samples was within 15%, and accuracy between –15 and 15%.

The stability experiments were performed to evaluate the stability of the analytes in human plasma under the following conditions: short-term stability at room temperature for 24 h; long-term stability at –80 °C for 30 days; three freeze (–80 °C)–thaw (room temperature) cycles on consecutive days. The extracted QC samples kept in the autosampler at 4 °C for 12 h were analyzed to evaluate post-preparation stability. All stability testing in plasma was determined by analyzing five replicates of QC samples at three concentration levels. The determined concentrations were compared with the nominal values.

2.6. Pharmacokinetic study

The clinical protocol was approved by Medical Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University prior to the study. Ten volunteers were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. The volunteers who submitted the agreements to attend this project were medically examined for the pharmacokinetics study of BOS and GPD. The subjects were required to abstain from taking any other drug for 7 days prior to the start of test. They were also demanded not to smoke or drink alcohol for 24 h before the beginning of the study until its end. All volunteers were received an oral dose of 125 mg BOS and 2 mg GPD with 200 mL water. Blood samples (3 mL) were collected into heparinized tubes before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24 and 48 h after oral administration. Blood samples were centrifuged at 4000 × g for 10 min and the plasma was separated and kept frozen at –80 °C until analysis.

2.7. Data analysis

Plasma concentration vs. time profiles were analyzed using DAS software (Version 2.0, Medical University of Wenzhou, China) to estimate the type of compartment model and pharmacokinetic parameters. Data were expressed as mean ± SD.

3. Results and discussion

3.1. Method development and optimization

The choice of mobile phase should be concerned based on the consideration of ionization efficiency before the analytes enter the MS/MS system in order to obtain nice resolution and high sensitivity. As for the choice of strong elution mobile phase, methanol and acetonitrile were considered as two candidates. Results showed that the responses of analytes with acetonitrile as the mobile phase were higher than those with methanol under ESI positive mode. To obtain the maximum sensitivity, we investigated the effects of pH with various mobile phases on the ionization efficiency. Both analytes and IS were found to have the highest response and the best peak shapes in the mobile phase containing 0.1% formic acid. The LC mobile phase was optimized with varying percentages of organic solvent and different modifiers in water to obtain high sensitivity. Analytes and IS were separated on an Acquity UPLC BEH C18 column with a gradient mobile phase consisting of acetonitrile and 0.1% formic acid. The whole separation of two analytes and IS was completed within only 1.5 min per sample. BOS, GPD and IS were eluted at about 0.57, 0.90 and 0.77 min, respectively.

Sample preparation is a key step for accurate and reliable UPLC–MS/MS assays. Two kinds of sample treatment procedures were evaluated, including protein precipitation and liquid–liquid extraction (LLE). LLE could prepare purified and concentrated samples and improve the sensitivity and robustness of the assay. Protein precipitation was often used for the preparation of biological samples with the advantages of saving time and simplicity. In this work, two methods, including a protein precipitation procedure and acetic ether extraction, were investigated and compared. Because of a shorter sample preparation time, protein precipitation was employed as the extraction method.

| Table 1 | Recovery and matrix effect of BOS, GPD and internal standards (n = 6). |
|---------|------------------|------------------|------------------|------------------|------------------|
|         | Recovery (%)      | Matrix effect (%)|
|         | Mean ± SD         | RSD (%)          | Mean ± SD         | RSD (%)          |
| BOS     |                  |                  |                  |
| 10      | 90.67 ± 3.93     | 4.33             | 103.83 ± 7.00    | 6.74             |
| 200     | 94.33 ± 1.75     | 1.86             | 94.67 ± 2.66     | 2.81             |
| 800     | 94.67 ± 2.58     | 2.73             | 102.33 ± 5.01    | 4.89             |
| GPD     |                  |                  |                  |
| 5       | 87.50 ± 2.43     | 2.78             | 96.17 ± 4.26     | 4.43             |
| 100     | 95.67 ± 2.25     | 2.35             | 97.18 ± 1.33     | 1.37             |
| 400     | 90.83 ± 2.32     | 2.55             | 98.33 ± 4.84     | 4.93             |
| IS      |                  |                  |                  |
| 50      | 92.36 ± 2.78     | 3.01             | 101.58 ± 5.42    | 5.34             |

| Table 2 | Precision and accuracy of method for the determination of BOS and GPD in human plasma (n = 6). |
|---------|------------------|------------------|------------------|------------------|------------------|
|         | Intra-day precision (n = 6) | Inter-day precision (n = 6) |
|         | Mean ± SD         | RSD (%)          | RE (%)           | Mean ± SD         | RSD (%)          | RE (%)           |
| BOS     |                  |                  |                  |
| 10      | 10.49 ± 0.86     | 8.21             | 4.85             | 9.83 ± 0.63      | 6.38             | −1.70            |
| 200     | 205.50 ± 8.83    | 4.29             | 2.75             | 208.17 ± 11.37   | 5.46             | 4.08             |
| 800     | 796.50 ± 15.57   | 1.95             | −0.44            | 813.17 ± 25.95   | 3.19             | 1.65             |
| GPD     |                  |                  |                  |
| 5       | 4.96 ± 0.18      | 3.62             | −0.87            | 5.15 ± 0.31      | 5.95             | 2.90             |
| 100     | 97.33 ± 3.72     | 3.83             | −2.67            | 98.83 ± 5.31     | 5.37             | −1.17            |
| 400     | 405.17 ± 11.27   | 2.78             | 1.29             | 406.83 ± 14.54   | 3.57             | 1.71             |
Table 3
Stability results of BOS and GPD in human plasma in different conditions (n=5).

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Concentration added (ng/mL)</th>
<th>Room temperature, 24h</th>
<th>Autosampler 4 °C, 12h</th>
<th>Three freeze-thaw</th>
<th>−80 °C, 30 days</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>RSD (%)</td>
<td>RE (%)</td>
<td>Mean ± SD</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>BOS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.82 ± 0.85</td>
<td>8.63</td>
<td>−1.80</td>
<td>9.82 ± 0.47</td>
<td>4.77</td>
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<tr>
<td>200</td>
<td>192.67 ± 9.33</td>
<td>4.84</td>
<td>−3.67</td>
<td>200.67 ± 11.48</td>
<td>5.72</td>
</tr>
<tr>
<td>800</td>
<td>826.36 ± 23.40</td>
<td>2.83</td>
<td>3.29</td>
<td>797.83 ± 28.77</td>
<td>3.61</td>
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<tr>
<td>GPD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.94 ± 0.37</td>
<td>7.36</td>
<td>−0.77</td>
<td>4.94 ± 0.33</td>
<td>6.75</td>
</tr>
<tr>
<td>100</td>
<td>103.83 ± 6.31</td>
<td>6.07</td>
<td>3.83</td>
<td>103.50 ± 8.69</td>
<td>8.31</td>
</tr>
<tr>
<td>400</td>
<td>399.17 ± 13.41</td>
<td>3.36</td>
<td>−0.21</td>
<td>396.67 ± 13.22</td>
<td>3.34</td>
</tr>
</tbody>
</table>

3.2. Specificity
(Fig. 2A) Blank plasma spiked with analytes and IS (Fig. 2B) and
human plasma obtained 1 h after oral administration of BOS and
GPD (Fig. 2C). As shown in Fig. 2A, there were no obvious endogenous
interferences under the described chromatographic conditions.

3.3. Linearity and sensitivity
The calibration curves were created by plotting the peak area
ratios of the various analytes to internal standard versus num-
ber concentration of the analytes standards. Both calibration curves
were regressed using a linear equation. A weighting factor of
1/n² was calculated as follows: BOS, y = (0.094x + 0.0423, r = 0.9986); GPD,
y = (0.1913x + 2.54, r = 0.9963). As shown in Fig. 2, the correlation coefficient (r) was
more than 0.99. The equation of the standard calibration curve was
regression analysis. The LOQ values were 10 ng/mL for BOS and 2 ng/mL for GPD, re-
spectively.
3.4. Recovery and matrix effect

The extraction recoveries and matrix effect of BOS and GPD ranged from 87.50 to 95.67% and 94.67 to 103.83%, respectively. The extraction recovery and matrix effect of IS were 92.36% and 101.58%, respectively. The detailed results are presented in Table 1. The matrix effect on the ionization of the analytes and IS was not obvious under these conditions. These data indicated that the sample preparation method was satisfactory and resulted in little appreciable matrix effect for the analytes and IS.

3.5. Precision and accuracy

The intra- and inter-day precisions and accuracies of low, medium and high QC levels of the analytes are summarized in Table 2. The assay values for both intra- and inter-day were found to be within the accepted variable limits. The intra- and inter-day precisions of the analytes were within the range of 1.95–8.21% and 3.19–6.38%, respectively. The inter- and intra-day accuracies of the analytes were within the range of −2.67 to 4.85% and −1.70 to 4.08%, respectively. The results showed that the method was accurate and precise for the determination of the two analytes in human plasma.

3.6. Stability

The stability of the two analytes in plasma was investigated by analyzing five replicates of QC samples at three concentration levels after short-term storage (room temperature, 24 h), at 4 °C for 12 h after preparation, three freeze–thaw cycles, and long-term storage (−80 °C, 30 days). The results were found to be within the assay variability limits (±15%). All results of stability tests are shown in Table 3.

3.7. Application of the method in a pharmacokinetic study

The validated UPLC–MS/MS method was applied successfully in a single dose pharmacokinetic study of a combination dose of 125 mg BOS and 2 mg GPD in healthy Chinese subjects. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University. All subjects signed their informed consent form before the study. All the clinical samples were assayed by the validated method. The plasma samples with analyte concentrations above the upper limit of quantitation were diluted with blank human plasma. Representative chromatograms for BOS and GPD in real subject samples are shown in Fig. 2C. The mean plasma concentration time profiles of BOS and GPD in subjects are presented in Fig. 3. In addition, the main pharmacokinetic parameters are listed in Table 4.

4. Conclusions

An UPLC–MS/MS method for the simultaneous determination of BOS and GPD in human plasma was developed and validated. To the best of our knowledge, this is the first report of the simultaneous determination of BOS and GPD levels in human plasma using an UPLC–MS/MS method. The method affords the sensitivity, accuracy and precision needed for quantitative measurements of BOS and GPD in human plasma. It was also successfully applied in a clinical pharmacokinetic study.

References


